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13. ABSTRACT (Maximum 200 Words)

Retinoic acid (RA) has been used successfully in cancer prevention and therapy. RA exerts its biological effects through retinoic acid receptors (RARs, α, β, γ) It has been reported that RARβ plays an important role in mediating growth inhibitory actions of RA. The expression of RARβ is lost in prostate cancer cell lines, PC-3, and DU-145, while transfection of RARβ into PC-3 cells results in an increased sensitivity to growth inhibitory effects of RARβ aganist. Despite the correlation between the level of RARβ and the RA-associated growth inhibition, it remains unknown how RARβ mediates the growth inhibitory effects of RA. This study used murine F9 wild type (Wt) and RARβ knockout (F9 RARβ$^{-/-}$) cells as an experimental model to investigate the molecular mechanisms by which RARβ mediates the growth inhibitory actions of RA. Our study demonstrated that p27, a cell cycle progression regulatory protein, is increased by RA in F9 Wt cells as compared to the F9 RARβ$^{-/-}$ cells. In addition, RA stabilizes the protein stability of p27. Considering the striking findings that transfection of RARβ into the PC-3 cells results in an increased sensitivity to growth inhibition caused by RARβ against, our study may lead to more efficient chemotherapy with retinoids.

14. SUBJECT TERMS
Prostate, retinoic acid, retinoic acid receptor, p27

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Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer deaths in males in the United States. Retinoids (retinol and its metabolites and derivatives) have been used in the prevention and treatment of some types of cancer. It has been shown that retinoic acid (RA), a biologically active form of retinol, is effective in inhibiting the cell growth and promoting differentiation of prostate cancer. It exerts its biological activities by binding to nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). There are three RARs and three RXRs encoded by different genes (α, β, γ). Each RAR and RXR gene encodes several protein isoforms, generated by different promoter usage or alternate splicing. The RARβ_2 isoform, the most abundant RARβ isoform, is transcriptionally induced by RA in many cell types (1). A limitation to designing effective retinoid therapies in the treatment of prostate cancer is the lack of understanding of the molecular mechamisms that control retinoid-mediated growth inhibition and differentiation. It has been reported that prostate cancer cell lines PC-3 and DU-145 do not express RARβ, while stable expression of RARβ into the PC-3 cells results in an increased response to growth inhibition mediated by a RARβ aganist and a hexafluoride vitamin D3 analog (2). There are data indicating that RARβ plays an important role in mediating the growth inhibitory actions of RA. Conversely, the loss of RARβ expression occurs during the process of carcinogenesis. Reduced expression of RARβ is a common feature of premalignant lesions and carcinogenesis. (3-21). Malignant cells with decreased expression of RARβ become resistant to RA treatment (15, 22, 23), whereas the up-regulation of RARβ parallels RA-induced growth suppression in some tumor cells (24-26). In this study we studied the mechanisms by which RARβ mediates the growth inhibitory actions of RA by using murine F9 wild type (F9 Wt) and F9 RARβ_2 knockout (F9 RAR$\beta_2^{-/-}$) cells as an experimental model.

Body

We have previously shown that the F9 teratocarcinoma RARβ_2 knockout cell line exhibits no growth arrest in response to RA, whereas F9 Wt, F9 RAR$\alpha^{-/-}$ and F9 RAR$\gamma^{-/-}$ cell lines do growth arrest in response to RA. To examine the role of RARβ_2 in growth inhibition, we analyzed the cell cycle regulatory proteins affected by RA in F9 Wt and F9 RAR$\beta_2^{-/-}$ cells. Flow microfluorimetry analyses revealed that RA treatment of F9 Wt cells increased the percentage of cells in the G1/G0 phase of the cell cycle. In contrast, RA did not alter the cell cycle distribution profile of RAR$\beta_2^{-/-}$ cells. In F9 Wt cells, cyclin D1, D3 and cyclin E protein levels decreased, while cyclin D2 and p27 levels increased after RA treatment. Compared to the F9 Wt cells, the F9 RAR$\beta_2^{-/-}$ cells exhibited lower levels of cyclins D1, D2, D3, and E in the absence of RA, but did not exhibit further changes in the

levels of these cell cycle regulators after RA addition. Since RA significantly increased the level of p27 protein (~ 24-fold) in F9 Wt as compared to the F9 RAR$\beta_2^{-/-}$ cells, we chose to study p27 in greater detail. The p27 protein plays a pivotal role in the regulation of the proliferation and differentiation of many cell types. Down-regulation of p27 has been observed in carcinogenesis and metastasis and the level of p27 has been used to evaluate cancer progression (27). The p27 mRNA level and the rate of p27 protein synthesis were increased in RA treated F9 Wt cells, but not in F9 RAR$\beta_2^{-/-}$ cells. Moreover, RA increased the half-life of p27 protein in F9 Wt cells. Reduced expression of RARβ_2 is associated with the process of carcinogenesis and RARβ_2 can mediate the growth arrest induced by RA in a variety of cancer cells. Using both genetic and molecular approaches, we have identified some of the molecular mechanisms, such as the elevation of p27, through which RARβ_2 mediates these growth inhibitory effects in F9 cells.

RA Results in Cell Growth Arrest in F9 Wt but not in F9 RARβ2$^{-/-}$ Cells

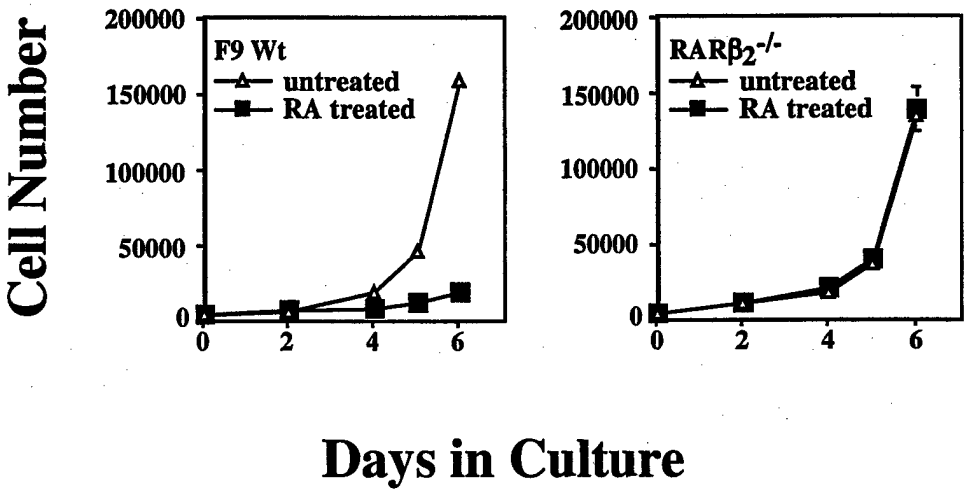


Figure 1A. Analysis of the growth of F9 Wt and RARβ2-/- cells after treatment with 1 μM RA. The cells were plated in duplicate wells at a density of 3000 cells/well. The cell numbers were counted on the indicated days. The experiment was performed three times with very similar results. The values represent the mean ± S.D. of three independent experiments.

RA Increases the Percentage of Cells in G1 Phase in F9 Wt but not in F9 RARβ$_2^{-/-}$ Cells

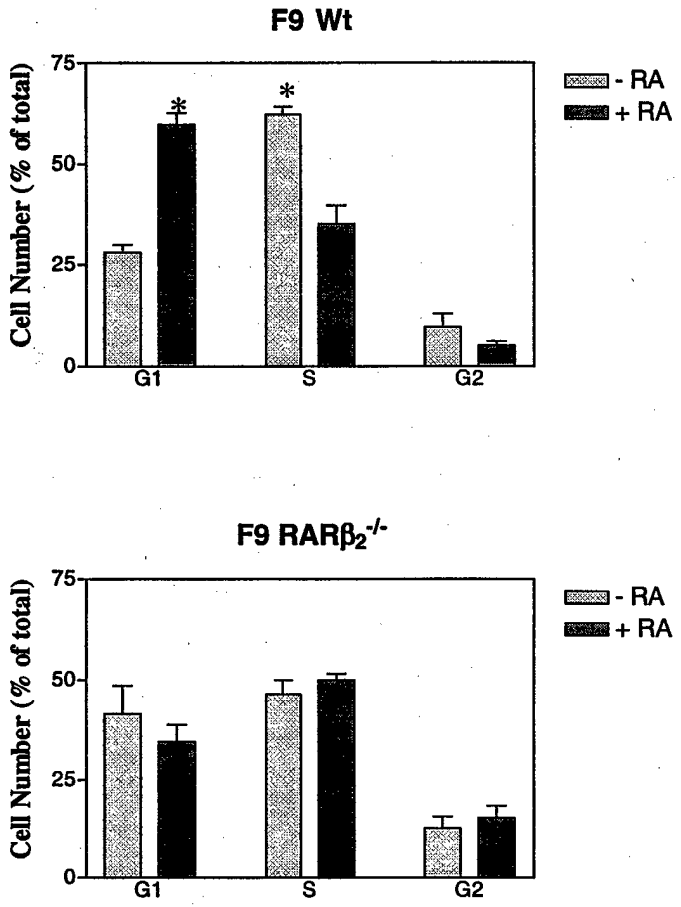


Figure 1B. **Statistical analysis of the cell cycle distribution of F9 Wt and RARβ$_2^{-/-}$ cells after treatment with 1 μM RA for 96 hours. The values represent the mean ± S.D. of three independent experiments. * $P < 0.05$.**

RA Altered Cell Cycle Regulatory Proteins in F9 Wt and F9 RARβ2$^{-/-}$ Cells

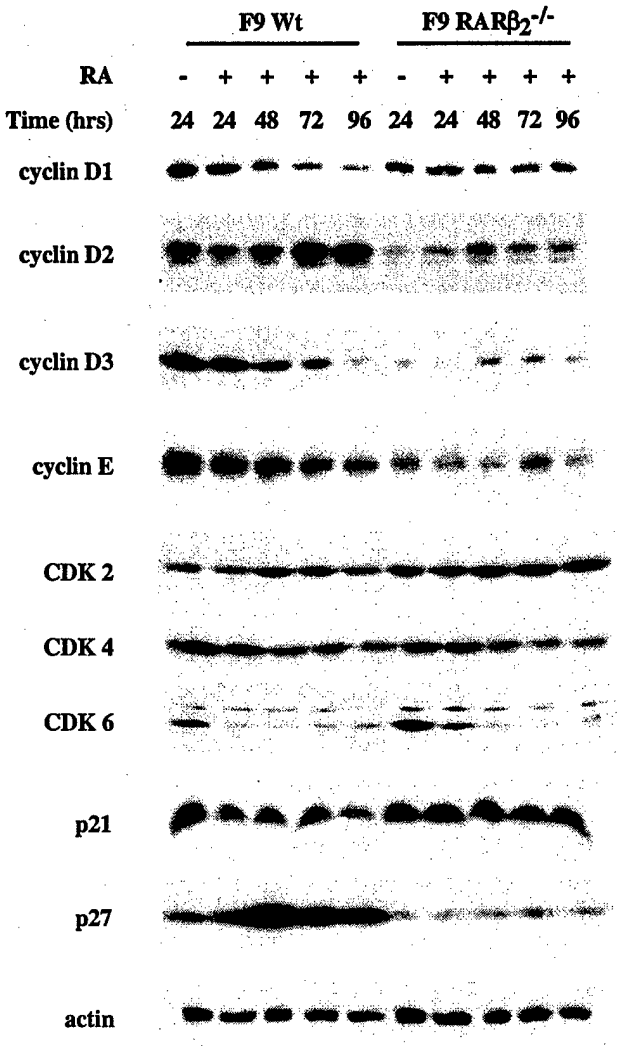
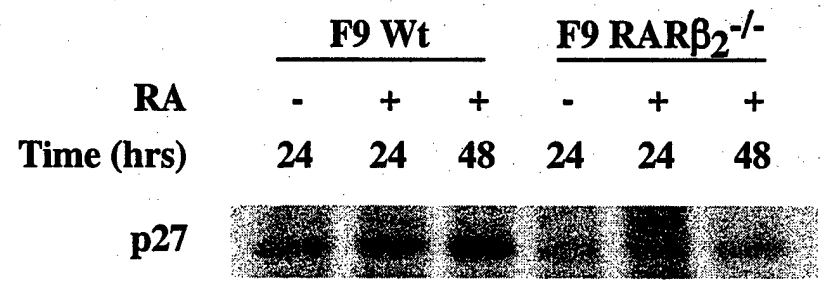


Figure 2. F9 Wt and RARβ2-/- cells were treated with 1 μM RA for the times indicated. Total cell lysates were prepared and Western blot analysis was performed. The experiment was performed three times with each antibody with similar results. Actin was used as a loading control.

RA Increases the Synthesis of p27 Protein in F9 Wt but not in F9 RARβ$_2^{-/-}$ Cells

A



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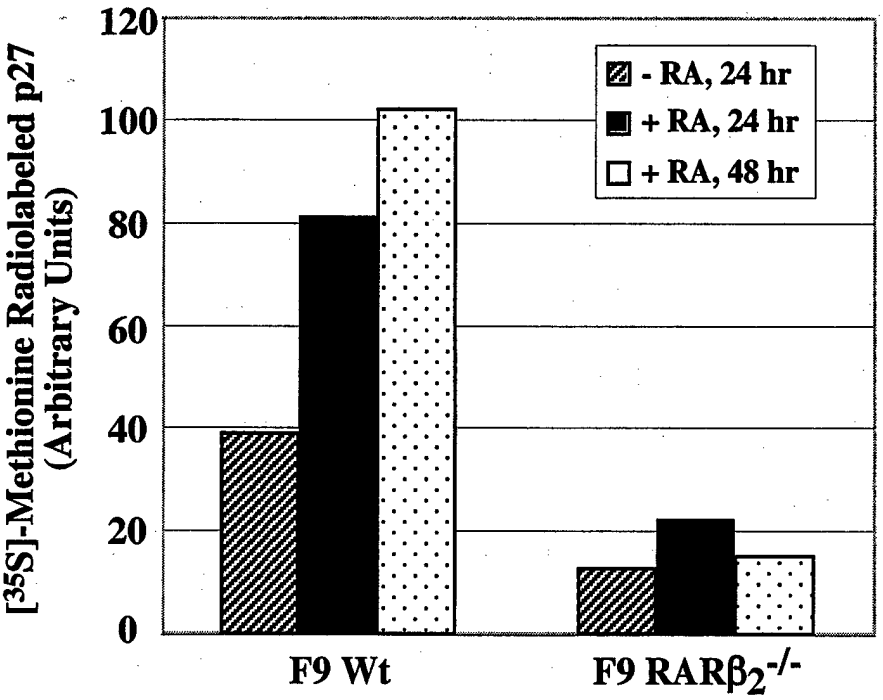
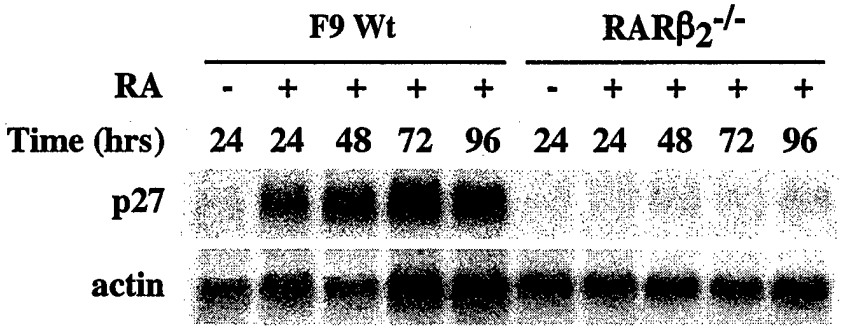


Figure 3. (A) F9 Wt and RARβ2-/- cells were treated with 1 μM RA for the times indicated and then labeled with 50 μCi/ml [35S]-methionine for 30 minutes. Immunoprecipitation with anti-p27 antibody was performed. The radiolabeled protein precipitates were electrophoresed on a 10% SDS-polyacrylamide gel that was subjected to autoradiography. (B) The amount of signal in A was quantified by NIH Image. The experiment was performed three times with very similar results.

RA Increases the Level of p27 mRNA in F9 Wt but not in F9 RARβ2$^{-/-}$ Cells

A



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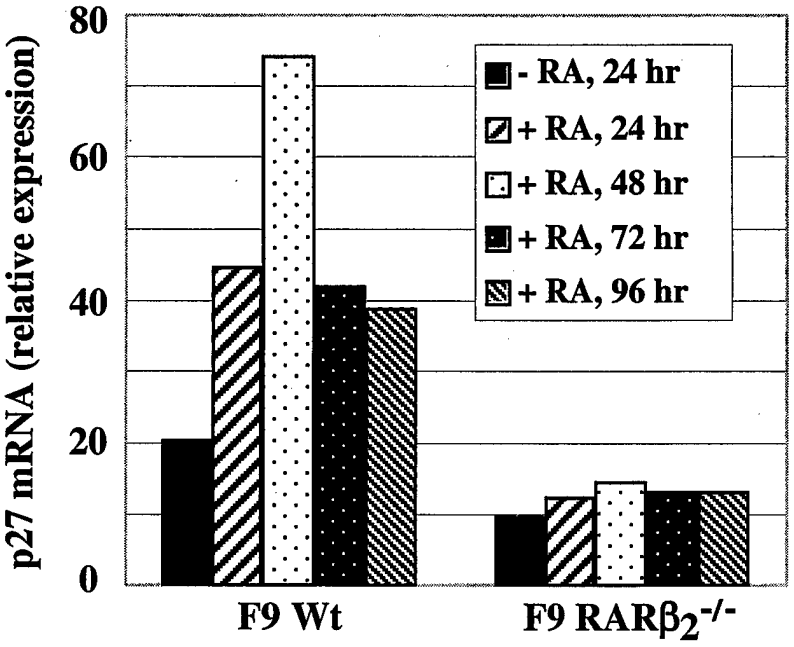

Figure 4. (A) F9 Wt and RARβ2-/- cells were treated with 1 μM RA for 96 hours. Total cellular RNA was extracted and Northern blot analysis was used to detect the level of p27 mRNA. (B) The amount of signal in A was quantified by NIH Image. The relative expression level of p27 mRNA was depicted as the ratio of the density of p27 mRNA to actin mRNA for the same time point. This experiment was performed three times with very similar results.

RA Stabilizes p27 Protein in F9 Wt Cells

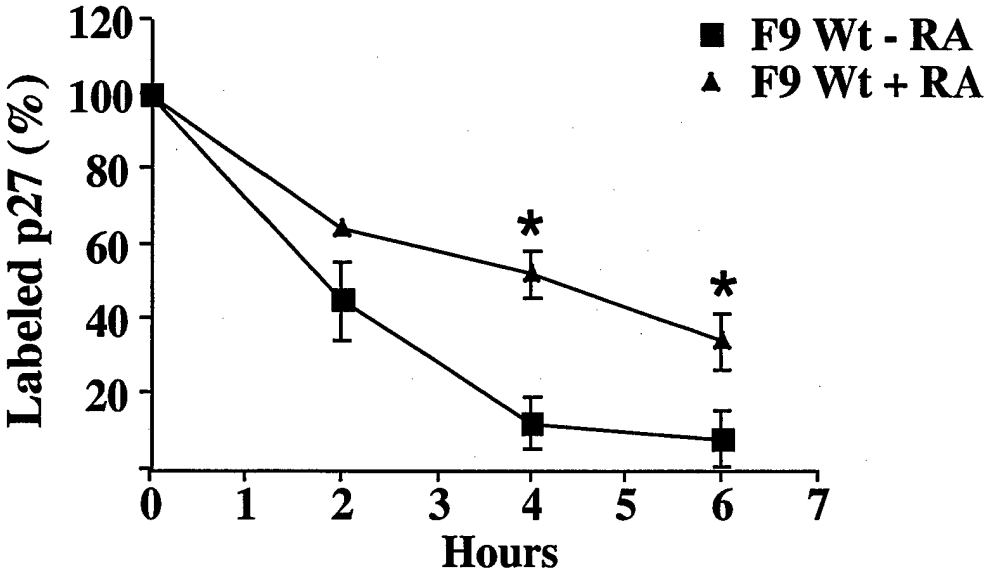

Figure 5. F9 Wt cells were cultured in the absence or presence of 1 μM RA for 48 hours, pulse-labeled with 100 μCi/ml [35S]-methionine in the presence or absence of RA for 1 hour and chased for 6 hours. Immunoprecipitation with anti-p27 antibody was performed. The protein precipitates were electrophoresed on a 10% SDS-polyacrylamide gel that was subjected to autoradiography. The amount of p27 in the absence or presence of RA was analyzed with ImageQuant. The amount of p27 in the absence or presence of RA immediately after the 1 hour pulse labeling is set at 100. * $P < 0.05$.

Key Research Accomplishments

- Examined the effects of RA via RARβ on the protein levels of several cell cycle regulatory porteins, one of which is p27.
- Investigated the effects of RA via RARβ on the levels of p27 mRNA and protein.
- Determined the effects of RA via RARβ on the stability of p27 protein.

Reportable Outcomes

Delineated some of the molecular mechanisms by which RARβ mediates the growth inhibitory effects of RA.

Conclusions

The increase of p27 is associated with the growth inhibition induced by RA via RARβ in F9 Wt cells. Considering the striking findings that stable transfection of RARβ to PC-3 cells results in a sensitivity to growth inhibition caused by RARβ against and a vitamin D3 analog, these data may be of use in designing more efficient chemotherapy with retinoids.

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In addition to what I previously reported, I studied the effects of retinoids and lecithin:retinol acyltransferase (LRAT) on the differentiation of human prostate cancer cells. LRAT is an enzyme involved in the metabolism of retinol to retinyl esters. It has been reported that the levels of LRAT and retinyl esters are reduced in some human cancers, such as prostate. The human prostate cancer cell line PC-3 was transfected with LRAT. The functional activity of LRAT in all the transfected cell lines was determined by HPLC (Figure 1). All the transfected cell lines took up and esterified retinol into retinyl esters, while PC-3 wild type cells did not. The PC-3 and PC-3/LRAT transfectant cells were treated with retinoic acid (RA) or retinol (ROL) for various times. RT-PCR was used to test the effects of retinoids and LRAT on several molecular markers of retinoid action, such as keratin 18 and Gbx2, in human prostate. Soft agar assays for tumor cell growth were also performed.

Our data showed that there were no obvious changes in the levels of the above molecular markers upon RA or ROL treatment in both PC-3 and PC-3/LRAT transfactant cells (Figure 2). These findings are important both in basic and clinical research. They indicate that retinyl esters are not crucial ligands for the regulation of the above genes in the carcinogenesis of human prostate. Our studies provide new information about retinoid effects on prostate cancer cells and provide a rationale for more efficient chemotherapy with retinoids.

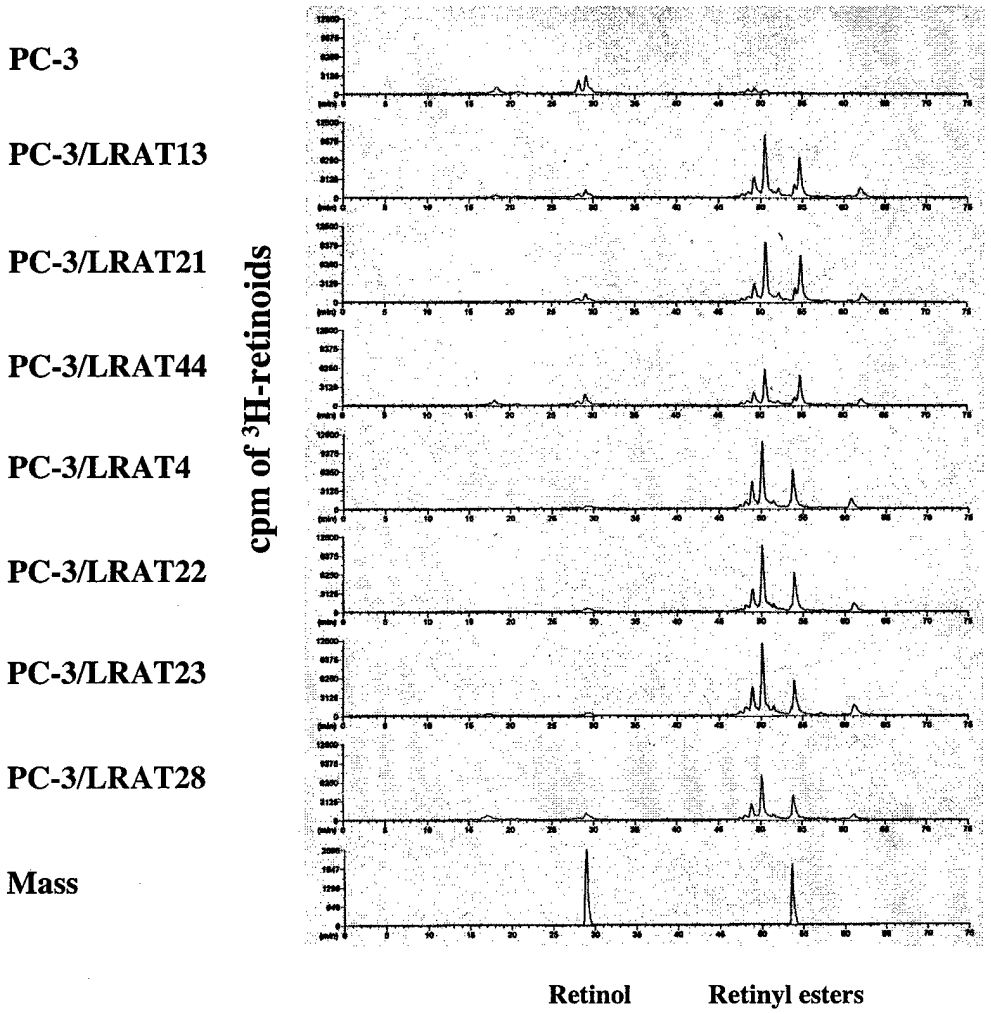

Figure 1. Retinol esterification in PC-3 wild type and PC-3/LRAT transfectants.

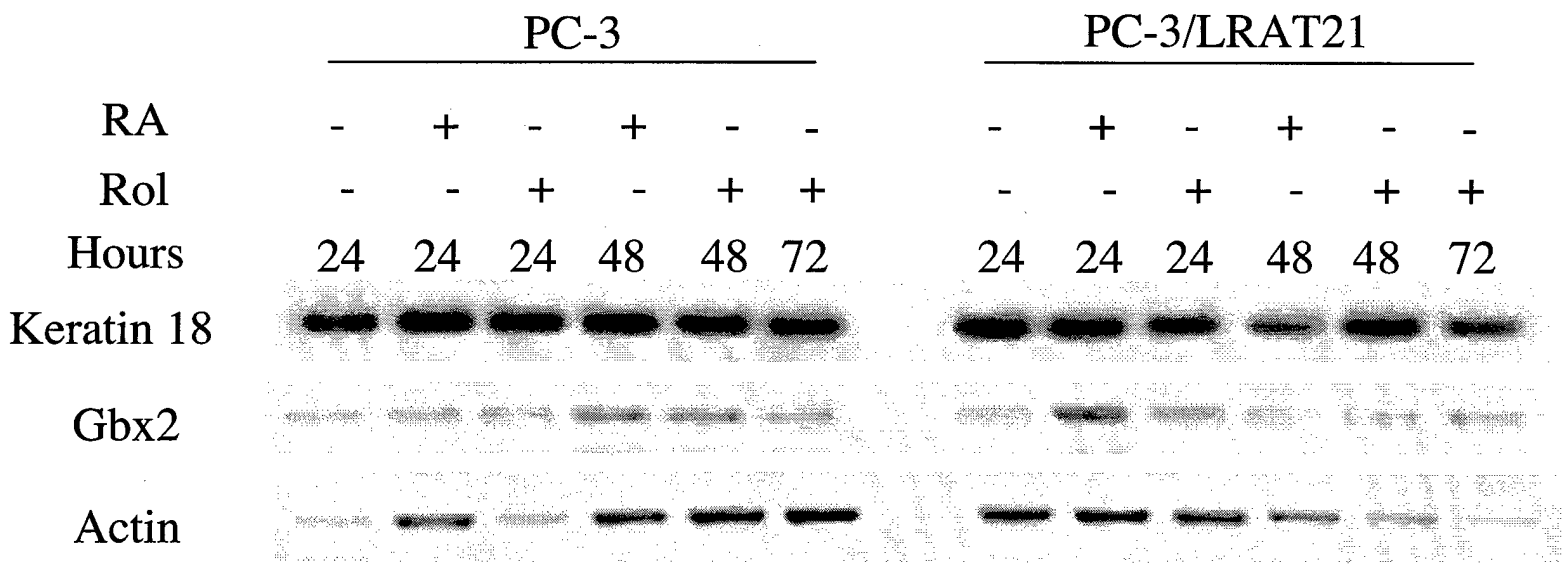

Figure 2. The effects of LRAT on the differentiation markers of human prostate.